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Award Number: DAMD17-02-1-0286

TITLE: A Unique Breast Cancer Cell Model for Studying Reported
Functions of Membrane-Localized Estrogen Receptor

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REPORT DATE: April 2004

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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20040830 079

REPORT DOCUMENTATION PAGEForm Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE April 2004	3. REPORT TYPE AND DATES COVERED Annual (1 Apr 2003 - 31 Mar 2004)	
4. TITLE AND SUBTITLE A Unique Breast Cancer Cell Model for Studying Reported Functions of Membrane-Localized Estrogen Receptor			5. FUNDING NUMBERS DAMD17-02-1-0286	
6. AUTHOR(S) Adrian V. Lee, Ph.D.				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Baylor College of Medicine Houston, Texas 77030-3498 <i>E-Mail:</i> avlee@breastcenter.tmc.edu			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES Original contains color plates: All DTIC reproductions will be in black and white.				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited				12b. DISTRIBUTION CODE
13. ABSTRACT (Maximum 200 Words) We previously developed a cell line system in which exogenous expression of estrogen receptor alpha (ER α) in an ER α -negative cell line resulted in ER α -mediated signaling and proliferation. We have now generated cell lines that express ER α only in the cytoplasm (cER α) to characterize the putative cytoplasmic (non-genomic) function of ER α . We have found that the cER α can bind estrogen and is down-regulated, similar to wild-type ER α . Intriguingly, the cER α is completely resistant to ICI182780-mediated degradation. cER α can't activate gene transcription (due to its inability to enter the nucleus), and also can't stimulate cell cycle progression. Consistent with the cER α not activating gene transcription or cell cycle progression, cER α is not able to induce ER-regulated genes. Despite evidence that in some systems that estrogen can have short-term rapid signaling events, we do not find that expression of cER α increases short-term signaling in C4-12 cells. We are currently using a rhodopsin-tagged ER which is in the plasma membrane to determine if membrane association is critical for short-term signaling, and also whether cER α can increase short-term estrogen signaling in MCF-7 cells that overexpress HER2.				
14. SUBJECT TERMS Estrogen receptor alpha, breast cancer, cyclin D1, IRS-1				15. NUMBER OF PAGES 9
				16. PRICE CODE
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

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INTRODUCTION

Since the discovery of membrane estrogen receptor α (ER α) more than 20 years ago, reports on this form of ER α signaling have continued to be documented and have recently received increasing attention. However, this field remains very controversial with nuclear ER α action being studied in much greater detail and becoming much better understood. The IDEA of this proposal is to create a novel and unique model of breast cancer cells that express only cytoplasmic or membrane estrogen receptor (and not nuclear ER α) and then compare and contrast ER α action to cells that express no ER α or wild-type ER α . We are in a unique situation to perform this, as we have recently shown for the first time that ER can be functionally expressed and regulate proliferation in an ER α -ve breast cancer cell line (C4-12).

BODY

Summary

This progress report is for year 2 of the project. In the first year of the project we made significant progress, having generated stable transfectants that express ER α in the cytoplasm (cER α) and having performed a preliminary characterization of these cells. However, as described in the body of this report, in the second year, we were unable to identify any increase in rapid estrogen signaling in these cells that expressed the cER α . This suggests that either these cells are not suitable for studying rapid estrogen effects or that perhaps the ER α needed to be in or near the plasma membrane to signal in this manner. In the second year we also struggled to create an ER variant that would reside in the plasma membrane. We expect that the small localization tag (myr or CAAX), placed on the N or C-terminus of ER α is folded within the protein and inaccessible for attachment to the plasma membrane. However, we now have a construct, received from a collaborator (Dr Wang, Johns Hopkins University), which is ER α linked to the membrane protein rhodopsin. We show data here that this construct gives membrane localization of ER α and will use this construct in year 3. In year 3 we will also examine the effect of cER α and rho-ER α on rapid estrogen signaling in breast cancer cells that overexpress HER2 and have been shown to exhibit short-term estrogen signaling via MAPK.

Task 1: To create and characterize ER α -negative MCF-7 cells (C4-12) that stably express GFP tagged membrane ER α (mER α), cytoplasmic ER α (cER α), or wild type (wtER α) (Months 1-12):

i) *Stably transfect ER α -negative MCF-7 (C4-12) cells with GFP, GFP-wtER α , GFP-mER α and GFP-cER α , and select cell lines that have low and high levels of the receptor (Months 1-6).*

We have stably transfected C4-12 cells with GFP, GFP-wtER α , and GFP-cER α . We have isolated multiple clones and using immunofluorescence microscopy shown that the cER α is indeed expressed in the cytoplasm (compared to wtER α which is mainly nuclear) (Figure 1). GFP alone is expressed all over the cell.

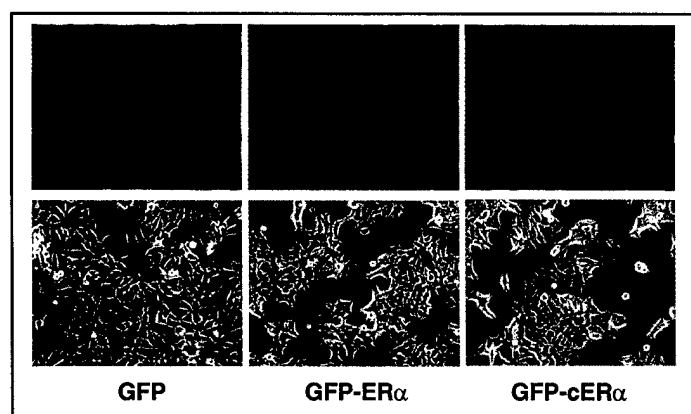


Figure 1: Stable expression of GFP-cER α in C4-12 cells. cER α was generated by deletion of the nls (245-270aa) in ER α . Stable clones of GFP, GFP-wtER α , and GFP-cER α were obtained and visualized by fluorescence microscopy (top panels) or by phase contrast (lower panels). GFP was widely distributed over the cell. In contrast, wt-ER α was exclusively nuclear; however, cER α was again widely distributed over the cell and did not show nuclear localization.

We have confirmed that GFP-cER α does not show nuclear localization by biochemical fractionation (data not shown) and confocal microscopy (Figure 2).

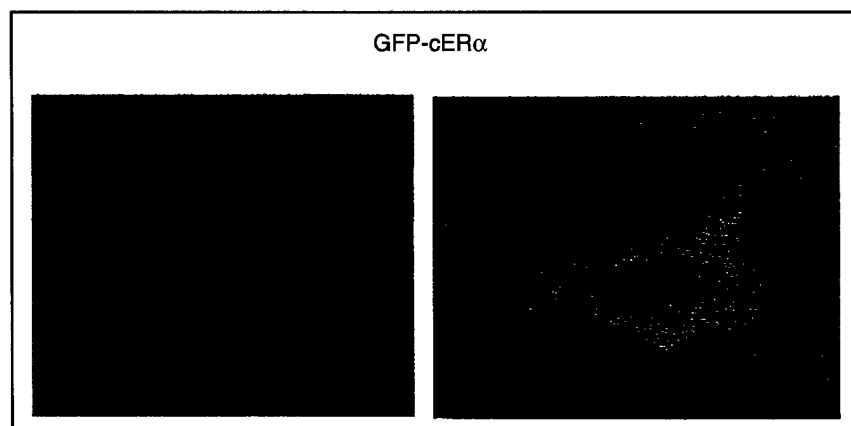


Figure 2: GFP-cER α is exclusively cytoplasmic. C4-12 cells stable expressing GFP-cER α were stained with propidium iodide (red) and then examined by confocal microscopy. The GFP signal shows that the cER α is exclusively cytoplasmic.

A setback came however, when we tried to express mER α , which was not targeted to the membrane (data not shown). Tagging of ER α with either C or N-terminal membrane signals does not send ER α to the membrane. This is probably due to folding of the protein making the tag inaccessible. We have therefore entered into collaboration with Dr Wang from Johns Hopkins University. He generated an ER α construct that consists of rhodopsin linked to ER α . Rhodopsin is membrane bound and so directs ER α to the plasma membrane (Xu Y *Mol Endocrinol* 2004 Jan, 18:86-96.) We found the rho-ER α to be in the endoplasmic reticulum following transient transfection with high concentrations of DNA (data not shown), however, lower amounts of DNA caused the rho-ER α to give only a plasma membrane signal (Figure 3).

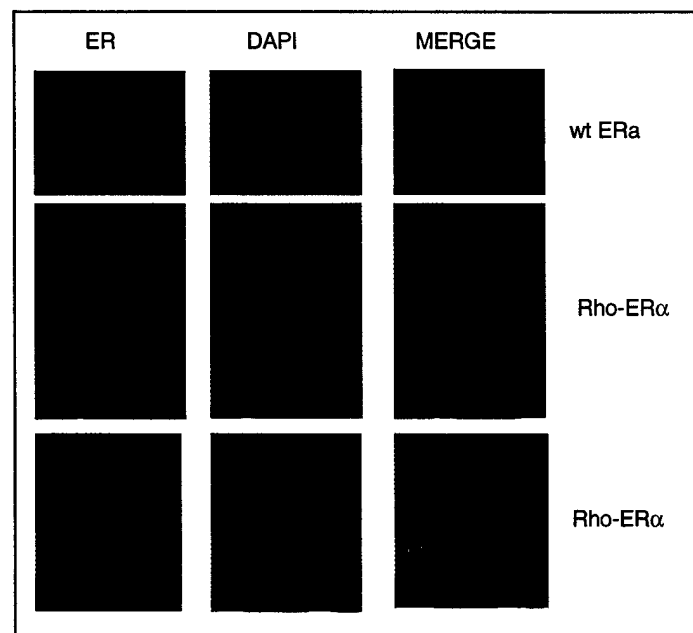


Figure 3: Localization of rho-ER α . 293 cells were transiently transfected with wt-ER α or rho-ER α and then ER α detected by immunostaining with ER α antibody (6F11, neomarkers) and detected by anti-mouse antibodies conjugated to alexa488 (left panels). The nucleus was identified by staining with DAPI (middle panels). A merged image is also shown (right panels). As expected, wtER α was entirely nuclear, whereas the rhoER α construct showed exclusive cytoplasmic or membrane staining. We have not confirmed that this is solely membrane staining by confocal microscopy; however, the staining is clearly very different to cER α , and looks very similar to GFP that has a membrane tag on it (data not shown)

Dr Wang has recently published using this rhoER α construct, but all of these studies were performed in HEK293 cells transiently transfected with the rho-ER α . We believe it is important to determine the relevance of these studies in breast cancer cells, and in particular in cells by stable transfection. We are currently therefore stably transfecting this construct into C4-12 cells to see the effect of mER α on rapid estrogen signaling.

ii) Use biochemical fractionation and confocal microscopy to determine whether mER α and cER α are expressed only in the membrane and cytoplasm respectively, and test whether mER α and cER α are capable of binding estradiol (E2) and tamoxifen (Tam) (Months 4-8).

Figure 1 confirms that cER α is only expressed in the cytoplasm, while wt-ER α is mainly nuclear. Figure 2 shows that rho-ER α is mainly membranous.

We have not tested directly whether cER α can bind E2 or tam, however an indirect measure is the ability of E2 to downregulate the receptor (which occurs after E2 binding). We found that cER α is degraded following E2 stimulation, suggesting in an indirect way that this receptor can bind E2.

iii) Examine whether mER α or cER α associate with membrane or cytoplasmic structures (e.g. clathrin-coated pits) (Months 7-12).

We have not yet performed these assays. These will be done when the rho-ER α stable transfectants are made.

Task 2: To compare and contrast the effects of estrogen in C4-12-cER α , mER α and wtER α cells (Months 12-24):

i) Analyze the effect of short (mins) and long-term (hours) E2 stimulation on localization, movement, and degradation of the different GFP-ER α variants (Months 12-16).

We have not examined localization and movement, but we have found that the cER α can be degraded by E2 (Figure 4).

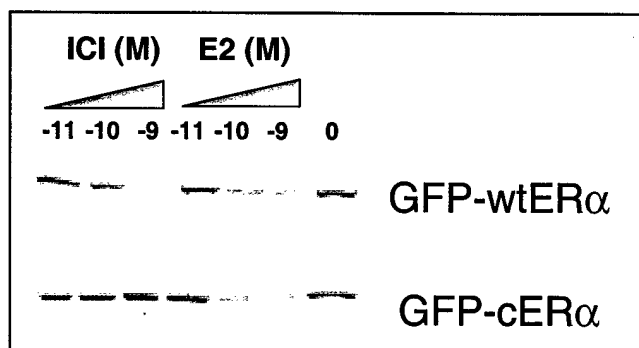


Figure 4: cER α is completely resistant to antiestrogen ICI 182780-mediated degradation. C4-12wt-ER α and cER α stable transfectants were starved in serum-free overnight and then treated for 8 hours with increasing concentrations of estradiol (E2) or ICI182780 (ICI). As expected, wtER α protein levels were reduced upon exposure to both E2 and ICI. This effect was blocked with the proteasome inhibitor (lactacystin 10uM) (data not shown). Of note, ICI required a 10-fold higher excess, which has previously been noted by others. In contrast to wtER α , cER α proteins levels decreased upon exposure to

E2, but were not affected by any concentration of ICI.

This is an important result given that a number of groups have proposed that E2-mediated degradation of ER α is linked to transcription. cER α is a variant ER that cant activate transcription, thus the degradation of cER by E2 represents a new paradigm for E2-mediated degradation of ER. Interestingly, while wt- ER α is degraded by antiestrogens such as ICI182780, the cER α is not degraded significantly by ICI182780 suggesting that this is a nuclear mediated event and that E2 and ICI degradation mechanisms are distinct. We are interested if membrane localization of rho-ER α will also inhibit degradation. We are currently cloning an SV40 nuclear localization sequence (nls) onto the cER α to put it back into the nucleus and see if it is then able to be degraded by ICI. This will determine whether the loss of ICI-mediated degradation in cER α is due to localization or due to the domain of ER α (240-275aa) that we have deleted.

ii) Examine whether ER-responsive genes (e.g. TGF α , PgR, cathepsin D, pS2, IRS-1, cyclin D1) are induced by E2 and inhibited by Tam (Months 15-20) by the different GFP-ER α variants.

We have found that cER α is incapable of inducing expression of genes such as IRS-1, IGF-IR, and cyclin D1 (Figure 5). This is consistent with it not being able to activate gene transcription in an ERE-luc reporter assay (data not shown).

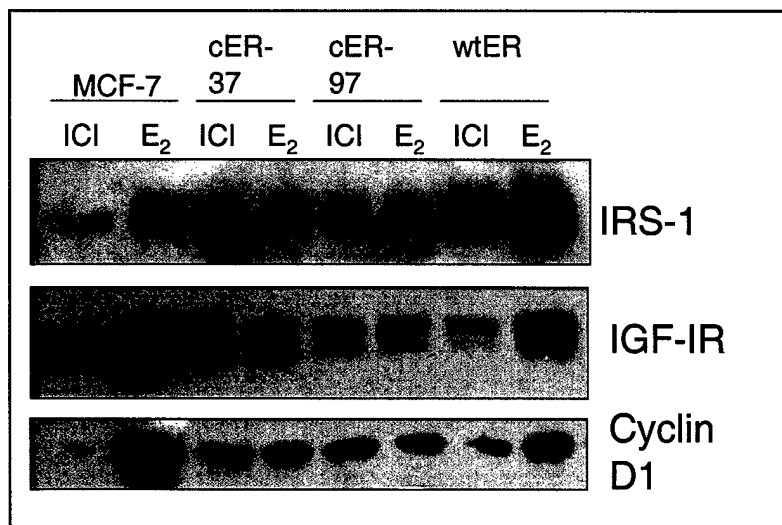


Figure 5: cER α doesn't confer estrogen-induction of ER-responsive genes. MCF-7, C4-12cER α and C4-12wtER α cells were starved in serum-free medium overnight and then treated with either antiestrogen (ICI, 10-9M) or estrogen (E₂, 10-9M) for 24 hrs. Cells were lysed in 5% SDS and immunoblotted with antibodies to insulin receptor substrate-1 (IRS-1), insulin-like growth factor receptor 1 (IGF-IR) or cyclin D1. As expected, all 3 genes were induced by estrogen in MCF-7 cells and also in C4-12 cells expressing wtER α . In contrast, two stable clones of C4-12ER α did not show estrogen regulation of IRS-1, IGF-IR or cyclin D1.

iii) Determine whether E₂ stimulation results in an increase in S-phase and cell proliferation in C4-12-cER α and mER α compared to C4-12wtER α (Months 18-24).

We have found that E₂ stimulation is able to increase S-phase in wt-ER α cells, but is unable to have an effect in cER α cells (Figure 6), consistent with this variant not inducing gene transcription (Figure 5). This is despite the fact that the cER α can clearly bind E₂ and be degraded.

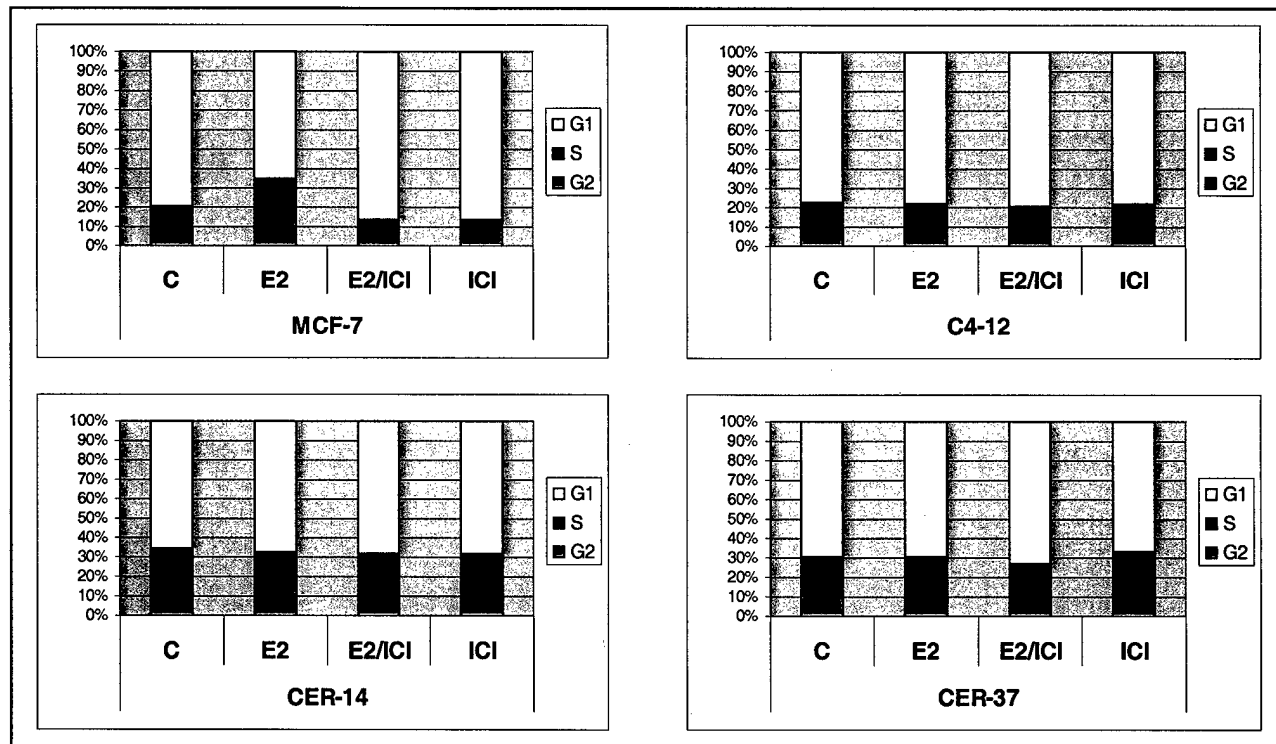


Figure 6: cER α does not confer estrogen stimulated S-phase entry. MCF-7, C4-12, and C4-12 cells expressing cER α were starved in serum-free medium overnight, and then stimulated with estradiol (1nM) or ICI (100nM) or the combination for 16hrs. Cells were then fixed in alcohol, stained with propidium iodide and FACS analysis performed. MCF-7 cells stimulated with estradiol showed an induction in S-phase fraction (red) and also an increase in cells entering G2/M (blue). These changes were completely blocked by ICI. In contrast, ER α negative C4-12 cells, or the cER α expressing cells showed no changes in response to E₂ or ICI.

Task 3: To determine whether previously reported short-term (minutes) E2-mediated effects are observed in C4-12-cER α or mER α cells (Months 24-36):

i) *Perform coimmunoprecipitation and colocalization to determine if mER α and cER α can bind p85 and activate PI3K (Months 24-30).*

Despite preliminary evidence that cER α was able to associate with p85, subsequent experiments failed to confirm an association with either p85 or IGF-IR. In addition we have been unable to show that cER can enhance short-term mediated activation of ERK1/2 or Akt by estradiol (Figure 7).

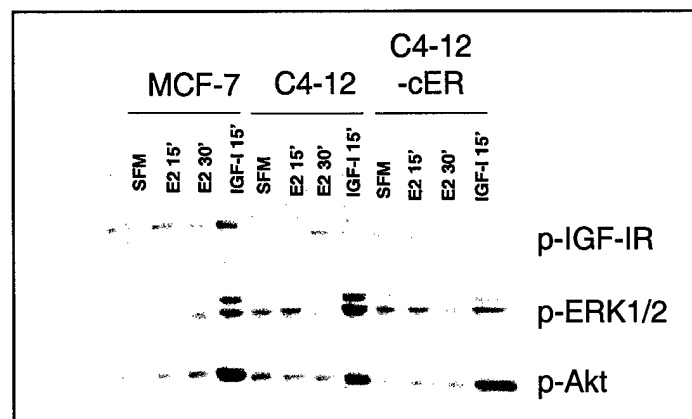


Figure 7: cER α does not allow short-term estrogen signaling in C4-12 cells. MCF-7, C4-12 and C4-12cER α cells were starved in SFM overnight and then stimulate with estradiol (1nM, 15 or 30mins) or IGF-I (10nM, 15mins). Cells were lysed and immunoblotted for p-IGF-IR, p-ERK1/2 and p-Akt. MCF-7 cells did not show a response to estradiol at 15 or 30mins. Similar results were also seen in both C4-12 cells. As a positive control, all 3 cell lines responded to IGF-I.

We do not know whether this result simply shows that cER α is not able to activate these pathways in these cells, or whether the specific system we chose is not suitable for detection of short-term estrogen effects. In this regard, members of our group have also recently failed to detect short-term estrogen signaling in MCF-7 cells, but have detected signaling in MCF-7 cells that overexpress HER2 (Figure 8 – kindly provided by Drs Osborne and Schiff, Breast Center, Baylor College of Medicine and in press in JNCI).

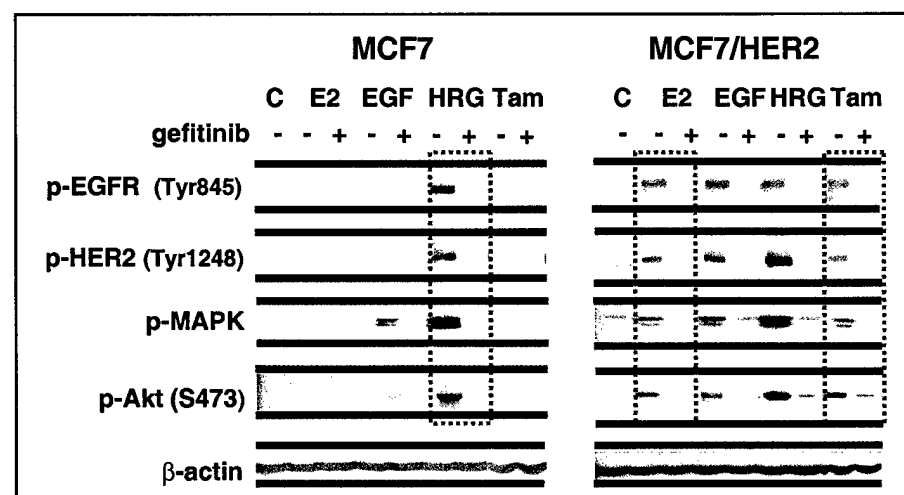


Figure 8: Rapid estrogen signaling in MCF-7 cells that overexpress HER-2 receptor. MCF-7 and MCF-7 cells stably overexpressing HER-2. Cells were starved overnight in serum-free medium and then stimulated for 15 minutes with estradiol (1nM), epidermal growth factor (EGF, 100nM), heregulin (HRG, 100nM) or tamoxifen (Tam, 100nM). These incubations were performed in the presence or absence of the EGFR kinase inhibitor Iressa (gefitinib). Cells were then lysed and immunoblotted for phospho-EGFR,

HER-2, Akt and ERK1/2 (MAPK). The left panel shows that estradiol or tam is unable to stimulate short-term signaling in MCF-7 cells, which can only be seen with heregulin (red box). In stark contrast, estradiol and tamoxifen were able to activate and phosphorylate all signaling intermediates in MCF-7/HER2 cells, and this was completely inhibited by EGFR blockade. We will therefore use these cells to better understand the role of cER α and rhoER α in this response. Figure kindly provided by Drs Osborne and Schiff, Breast Center, Baylor College of Medicine.

We have therefore decided to test the effect of HER-2 overexpression by two strategies. First we will examine the effect of cER α or rho-ER α overexpression on short-term estrogen action in MCF-7/HER2 cells. Second, we will overexpress HER-2 in C4-12 cells stably expressing the cER α or rho-

ER α . Increased HER-2 will be achieved by retroviral infection using a plasmid from Dr Schiff. In both systems we will then examine short-term estrogen effects

ii) *Examine whether E2-stimulation of C4-12-mER α and cER α cells results in mobilization of intracellular Ca²⁺ and activation of PKC (Months 30-34).*

Not performed yet due to lack of evidence in 3.i. Will be performed if the HER-2 experiments are successful at showing short-term estrogen signaling.

iii) *Determine whether E2 can induce an anti-apoptotic response in C4-12-mER α and cER α cells (Months 32-36).*

Not performed yet due to lack of evidence in 3.i. Will be performed if the HER-2 experiments are successful at showing short-term estrogen signaling.

KEY RESEARCH ACCOMPLISHMENTS

- Generation of C4-12 cells that express ER α only in the cytoplasm (C4-12- cER α).
- Evidence that cER α is degraded by estrogen but not by antiestrogen

REPORTABLE OUTCOMES

Development of cell lines:

C4-12-GFP

C4-12-GFP-cER α

CONCLUSIONS

This project will use a unique cell line model (C4-12) to test if cytoplasmic (cER α) or membrane targeted ER α (mER α) can perform signaling and mediated proliferation. This research is critical, as several recent studies have suggested that cER α or mER α is important, and pathologists only analyze nuclear ER α , which might misclassify a number of breast cancer patients. We have generated cells that express ER α only in the cytoplasm. We find that this receptor can't activate gene transcription or proliferation, despite the fact that the receptor is degraded by E2 and thus presumably can bind E2. However, this receptor is unable to stimulate short term estrogen events. We are currently stably transfecting a membrane variant of ER α (rho-ER α) to see if this can signal. We are also examining the effect of HER-2 overexpression on rapid Estrogen-events. Understanding any potential role of cER α or mER α is critical for the complete understanding of estrogen action and targeting in breast cancer.

PRESENTATION

2004 "Expression of non-nuclear ER in ER-negative breast cancer cells doesn't confer estrogen-stimulated growth". 14th Annual Breast Cancer Think Tank Meeting, St Kitts.